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Citation for published version:

Granneman, S, Lin, C, Champion, EA, Nandineni, MR, Zorca, C & Baserga, SJ 2006, 'The nucleolar protein Esf2 interacts directly with the DExD/H box RNA helicase, Dbp8, to stimulate ATP hydrolysis', *Nucleic Acids Research*, vol. 34, no. 10, pp. 3189-99. <https://doi.org/10.1093/nar/gkl419>

Digital Object Identifier (DOI):

[10.1093/nar/gkl419](https://doi.org/10.1093/nar/gkl419)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Nucleic Acids Research

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The nucleolar protein Esf2 interacts directly with the DExD/H box RNA helicase, Dbp8, to stimulate ATP hydrolysis

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Received April 18, 2006; Revised May 2, 2006; Accepted May 19, 2006

ABSTRACT

While 18 putative RNA helicases are involved in ribosome biogenesis in *Saccharomyces cerevisiae*, their enzymatic properties have remained largely biochemically uncharacterized. To better understand their function, we examined the enzymatic properties of Dbp8, a DExD/H box protein previously shown to be required for the synthesis of the 18S rRNA. As expected for an RNA helicase, we demonstrate that recombinant Dbp8 has ATPase activity *in vitro*, and that this activity is dependent on an intact ATPase domain. Strikingly, we identify Esf2, a nucleolar putative RNA binding protein, as a binding partner for Dbp8, and show that it enhances Dbp8 ATPase activity by decreasing the K_M for ATP. Thus, we have uncovered Esf2 as the first example of a protein co-factor that has a stimulatory effect on a nucleolar RNA helicase. We show that Esf2 can bind to pre-rRNAs and speculate that it may function to bring Dbp8 to the pre-rRNA, thereby both regulating its enzymatic activity and guiding Dbp8 to its site of action.

INTRODUCTION

In eukaryotes, members of the family of DExD/H box RNA helicases are involved in many different steps of RNA metabolism, from RNA processing and RNA export to translation. The activity of RNA helicases is generally associated with the separation of RNA strands ('unwinding'), hence the name RNA helicase. Recently, it has become evident that these proteins also have the capacity to anneal RNA strands or to remodel ribonucleoprotein (RNP) complexes by disrupting protein–RNA interactions (1–4). Interestingly, the latter

activity does not necessarily involve strand displacement (4). Thus, the term 'RNA helicase' is clearly a very simple description for the diverse biochemical properties of this family of proteins.

DExD/H box RNA helicases generally harbor 7–9 conserved motifs, which comprise the core RNA helicase domain [reviewed in (5,6)]. These motifs are not only involved in binding of the RNA substrate, but also are required for binding and hydrolysis of NTP, generally ATP, and use energy that is released in this reaction to drive the RNA/RNP remodeling steps. The recently discovered Q motif, unique to DEAD box proteins, has been proposed to function in ATP and RNA substrate binding (7,8). Motifs I, II and VI are responsible for binding of ATP and the Mg^{2+} co-factor, whereas motif III (SAT) is implicated in coupling ATP hydrolysis with RNA unwinding activity.

Because RNA helicases are involved in diverse steps in gene expression that require large RNA–protein complexes, it is likely that they are not acting alone but in association with other proteins. By the accounting of Silverman *et al.* in a recent review [Table 1 in (9)], there are at least 36 DExD/H box helicases that may have protein partners. Close inspection of this list indicates that only a few of the interactions have been characterized at the molecular level, and for even fewer have the biochemical parameters of the helicase been examined. Thus, the function of these putative co-factors in RNA helicase activity is largely unknown.

Ribosome biogenesis in yeast involves the synthesis of a 35S polycistronic rRNA precursor (pre-rRNA) that is processed in large pre-ribosomal particles at several well-defined sites to produce the mature 18S, 5.8S and 25S rRNAs [for a recent review see (10)]. This process requires over 200 proteins and hundreds of small nucleolar RNAs (snoRNAs), which function in cleavage and chemical modification of pre-rRNA via complementary base pairing interactions with the rRNA. A total of 18 putative RNA helicases are involved in pre-rRNA processing and ribosome assembly. Mutational

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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studies of RNA helicases involved in ribosome biogenesis revealed that conserved amino acids predicted to be involved in ATP binding and hydrolysis are important for cell viability, suggesting that they may indeed function as RNA helicases (11–13). Thus far, only four of the 18 proteins (Dbp4, Rok1, Dbp9 and Has1) have been characterized *in vitro* and have been shown to harbor ATPase activity (14–17). Two of these proteins (Dbp9 and Has1) were also able to separate artificial nucleic acid duplexes *in vitro*, suggesting that they are indeed helicases (14,17). However, biochemical evidence to support this hypothesis for the other 14 helicases is lacking.

We have begun to study the enzymatic properties of the RNA helicases involved in ribosome biogenesis in order to better understand their function. Here we present the biochemical characterization for the yeast DEAD box protein Dbp8, a putative RNA helicase involved in 18S rRNA synthesis (13). We demonstrate that purified recombinant Dbp8 exhibits ATPase activity *in vitro*, and that mutations in the predicted ATPase domain abrogate Dbp8 ATPase activity. Strikingly, we identify Esf2, a nucleolar RNA binding protein required for pre-18S rRNA synthesis (18,19), as a Dbp8-interacting protein. Using two-hybrid technology and with purified recombinant proteins, we show that Esf2 does indeed directly bind to Dbp8, likely via its C-terminal domain, and that this interaction substantially increases its ATPase activity. Thus, optimal ATP hydrolysis activity of Dbp8 depends on the activity of its protein co-factor, Esf2. Our results suggest a model in which Esf2 recruits Dbp8 to pre-ribosomes by binding to a region within the pre-rRNA. To our knowledge, Dbp8 is only the second known eukaryotic RNA helicase that requires a protein co-factor for optimal function.

MATERIALS AND METHODS

Yeast strains and media

YPH499 (mata, *ura3-52*, *lys2-80*, *ade2-101*, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) was the parental strain for the construction of strains used here. Strains carrying genes under the control of the galactose-inducible promoter or strains carrying triple HA-tagged genes (Kan^R marker) were generated as described previously (20). Strains expressing TAP carboxyl-tagged Dbp8 (*Kluyveromyces lactis* TRP marker) were generated as described previously (21). Unless specifically stated, strains were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) and *GAL::3HA* strains were grown in YPG/R (1% yeast extract, 2% peptone, 2% galactose and 2% raffinose) at 30°C. Strains harboring p415GPD::*ESF2* plasmids (*CEN* origin of replication, *LEU* marker) were grown at 30°C in SC—leucine (Clontech), supplemented with 2% dextrose or with 2% galactose and 2% raffinose.

DNA manipulations and *in vitro* RNA transcriptions

To over-express and purify six histidine-tagged Dbp8 protein, *DBP8* was amplified by PCR from genomic DNA and cloned into the pET28a expression vector using BamHI and NotI restriction sites. To over-express and purify glutathione *S*-transferase GST-Esf2, GST-Esf2 ΔC and GST-Rpa34, the alleles were amplified by PCR using genomic DNA as

template and the products were cloned into the pGEX6P-2 vector using BamHI, EcoRI and XhoI restriction sites. All constructs were verified by automated DNA sequencing (W.M. Keck DNA sequencing facility at Yale). For the yeast 2-hybrid experiments *ESF2* was PCR amplified and cloned into the pAS2-1 bait vector using NcoI and BamHI restriction sites. To clone *DBP8* into the prey vector (pACT2), the gene was amplified by PCR and cloned into pACT2 using SmaI and BamHI restriction sites. To generate *in vitro* transcribed rRNA fragments, rDNA templates containing a T7 promoter were constructed by PCR using yeast genomic DNA. The rRNA transcripts were generated using the MEGAscript kit according to the manufacturer's instructions (Ambion).

Expression and purification of recombinant proteins

The fusion proteins were expressed in *Escherichia coli* BL21 (DE3). Cells were grown in Luria–Bertani (LB) at 37°C supplemented with either kanamycin (pET28a) or ampicillin (pGEX) to an OD₆₀₀ of 0.4 and then grown for 1 h at 17°C. Protein expression was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 16–20 h at 17°C. Cells were harvested by centrifugation and pellets were resuspended in 5 vol of breaking buffer [150 mM KCl, 50 mM Tris–HCl (pH 7.4), 10% sucrose, 0.5 mM EDTA, 1 mM β-mercaptoethanol, 0.01% Igepal (Sigma)] containing a protease inhibitor cocktail (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 1.3 mM benzamidin; Sigma) and lysed using a Constant systems cell disrupter (Low March, Daventry Northants, UK) at 20 000 psi (1350 Bar). Extracts were clarified by ultracentrifugation in a Ti45 rotor at 100 000 g for 1 h. To purify recombinant six histidine-tagged (His6) Dbp8 proteins, clarified extracts prepared from ~25 g of cells was passed over a 30 ml SP Sepharose column pre-equilibrated with K100 buffer (like K1000 but with 100 mM KCl instead of 1 M). The SP Sepharose column was subsequently washed with 3 vol of K100 and bound proteins were eluted with an 80 ml linear salt gradient (100–1000 mM KCl) prepared in K-buffer [20 mM KHPO₄ (pH 7.4), 0.5 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol and 0.01% Igepal], followed by a 20 ml K1000 elution. Fractions enriched for His6-Dbp8 were pooled and the mixture was then incubated with 5 ml of Ni-NTA beads (Qiagen; 1 ml beads / 5 g of cell pellet) for 1 h at 4°C. The beads were then washed with 10 vol of K1000 followed by a wash with 10 vol of K300 containing 50 mM imidazole. Proteins were eluted in K300 containing 250 mM imidazole. Fractions containing nearly homogenous recombinant proteins were pooled and dialyzed for 4 h in K300 buffer containing 20% glycerol at 4°C.

To purify the GST-Esf2 wild-type and Esf2 ΔC fusion proteins, clarified extracts prepared from 10 g of cells were passed over 30 ml Heparin agarose columns (BioRad) and the fusion proteins were eluted by applying an 80 ml linear salt gradient (100–2000 mM KCl) prepared in K-buffer, followed by 20 ml K2000 elution step. Fractions enriched for the fusion proteins were pooled ([KCl] ~300 mM) and incubated with 4 ml of glutathione–Sepharose beads (Amersham) overnight at 4°C. After extensively washing the beads with K300 buffer, the proteins were eluted in K300 containing

200 mM reduced glutathione (USB). Fractions containing nearly homogenous recombinant proteins were pooled and extensively dialyzed in K300 buffer containing 20% glycerol at 4°C. Proteins were frozen in small aliquots in liquid nitrogen and stored at -80°C. The purity of the purified material was around 90% as judged by SDS-PAGE and Coomassie blue staining. Protein concentrations were determined using the Bradford assay as described by the manufacturer (BioRad).

ATPase experiments

Unless otherwise noted, ATPase assays were carried out using 10 pmol of recombinant His6-Dbp8 in a Tris-based buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM DTT, 1 µg BSA and 10 µM cold ATP (pH 7.0, Sigma) in a final reaction volume of 10 µl. Radioactive ³²P-γ-ATP was added as a tracer in order to quantify the amount of ATP converted to ADP. Reaction products were separated by thin layer chromatography and visualized via autoradiography. The ATP conversion rate was determined by quantifying ³²P release using a phosphorimager.

In vitro protein-protein and protein-RNA interactions

For the protein-protein interaction assays, 300 pmol of each protein was mixed in a K0 buffer [20 mM KHPO₄ (pH 7.4), 1 mM DTT and 0.1% Igepal] in a final volume of 100 µl. Unless otherwise noted, the potassium chloride concentration in the pull-down reactions was 150 mM. After 1 h incubation on ice, 100 µl of K150 containing 0.1% Igepal was added to the mixtures and 20 µl was removed for analysis (10% input). The remainder (180 µl) was subsequently incubated with 20 µl of glutathione-Sepharose beads (Amersham) for 2 h at 4°C. After this incubation step, 18 µl of the supernatant was removed for analysis (10% supernatant). The beads were subsequently washed three times with 500 µl of ice-cold K150 containing 0.1% Igepal. Bound proteins were eluted by boiling the beads in SDS-PAGE loading dye and resolved by 10% SDS-PAGE. Proteins were visualized by Coomassie brilliant blue staining.

For the protein-RNA interaction assays, 10 pmol of GST-fusion protein (diluted in K150) was incubated with 10 ng of *in vitro* transcribed and ³²P-labeled rRNA fragments in K0 buffer in a final volume of 10 µl. KCl was added to a final concentration of 150 mM. After 1 h incubation on ice, K150 was added to a final volume of 200 µl, 20 µl of the sample was removed (10% input) and the remainder (180 µl) was incubated with 20 µl of glutathione-Sepharose beads (Amersham) for 1 h on ice with regular agitation. The beads were subsequently washed three times with 500 µl of ice-cold K150 containing 0.1% Igepal. Beads were then resuspended in 300 µl K150 buffer and RNA was isolated by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. Co-precipitated RNAs were resolved on 6% polyacrylamide-8 M urea gels and visualized by autoradiography.

Miscellaneous

Yeast 2-hybrid, immunoprecipitation experiments and western blot analyses were performed as described elsewhere (22). Antibodies used: horseradish peroxidase (HRP)

conjugated mouse anti-His6 (Sigma). The 12CA5 anti-HA monoclonal antibody has been described elsewhere (22). Motif searches for Esf2 were carried out with Interpro (<http://www.ebi.ac.uk/interpro/>), SMART (<http://smart.embl-heidelberg.de/>), Prosite (<http://us.expasy.org/prosite/>) and the COILS program (http://www.ch.embnet.org/software/COILS_form.html), (23).

RESULTS

Recombinant Dbp8 has ATPase activity

The presence of the canonical DEAD box motifs in Dbp8 suggests that it may function as an ATPase and possess RNA unwinding activity. Recent studies have demonstrated that mutations at conserved amino acids within the putative ATPase motifs of Dbp8 are lethal, demonstrating that these motifs are essential for Dbp8 function *in vivo* (12,13). Moreover, over-expression of these mutants in a wild-type background causes dominant negative growth defects and delays in pre-rRNA processing as well as 18S rRNA synthesis (12,13). These combined results suggested that Dbp8 has ATP hydrolysis activity and that, furthermore, this activity is critical for 18S rRNA synthesis. However, biochemical evidence in support of this hypothesis was lacking. Thus, to gain more detailed insight into the biochemical properties of Dbp8 and its role in 18S rRNA synthesis, we have analyzed the ATPase activity of recombinant Dbp8 proteins *in vitro*.

To substantiate the *in vivo* results, we expressed and purified a six histidine-tagged Dbp8 protein (His6-Dbp8) from *E.coli* lysates (Figure 1A) and characterized its ATPase activity under a variety of conditions (Figure 1B-D). Our results indicated that Dbp8 had a strong preference for Mg²⁺ as metal co-factor (data not shown), was most active at 30°C (Figure 1B), had a pH optimum for activity of ~7.4 (Figure 1C) and a salt optimum of 300 mM KCl (Figure 1D). Much to our surprise, under the tested conditions, Dbp8 ATPase activity was not significantly stimulated by the presence of total yeast RNA, single-stranded or double-stranded DNA (data not shown). The presence of an excess of RNase A in the ATPase assay did not noticeably affect Dbp8 ATP hydrolysis activity and Dbp8 purified from RNase A treated *E.coli* extracts showed significant ATP hydrolysis activity (data not shown). Thus, under the tested conditions (pH 7.4, 300 mM KCl, 30°C, 5 mM MgCl₂) Dbp8 ATP hydrolysis activity does not require an RNA co-factor.

Conserved amino acids within the ATPase motifs are essential for Dbp8 ATPase activity *in vitro*

Mutations in the putative ATPase motifs (Motif I and II; K52A, K52R and D157A) are lethal *in vivo* and, when over-expressed, delay pre-rRNA processing and synthesis of the 18S rRNA (12,13). To correlate these *in vivo* results with the biochemical properties of Dbp8, we addressed whether these mutated proteins were capable of hydrolyzing ATP *in vitro* (Figure 2A). Mutant Dbp8 proteins were expressed and purified as described above and the quality of the protein preparations was determined by SDS-PAGE followed by

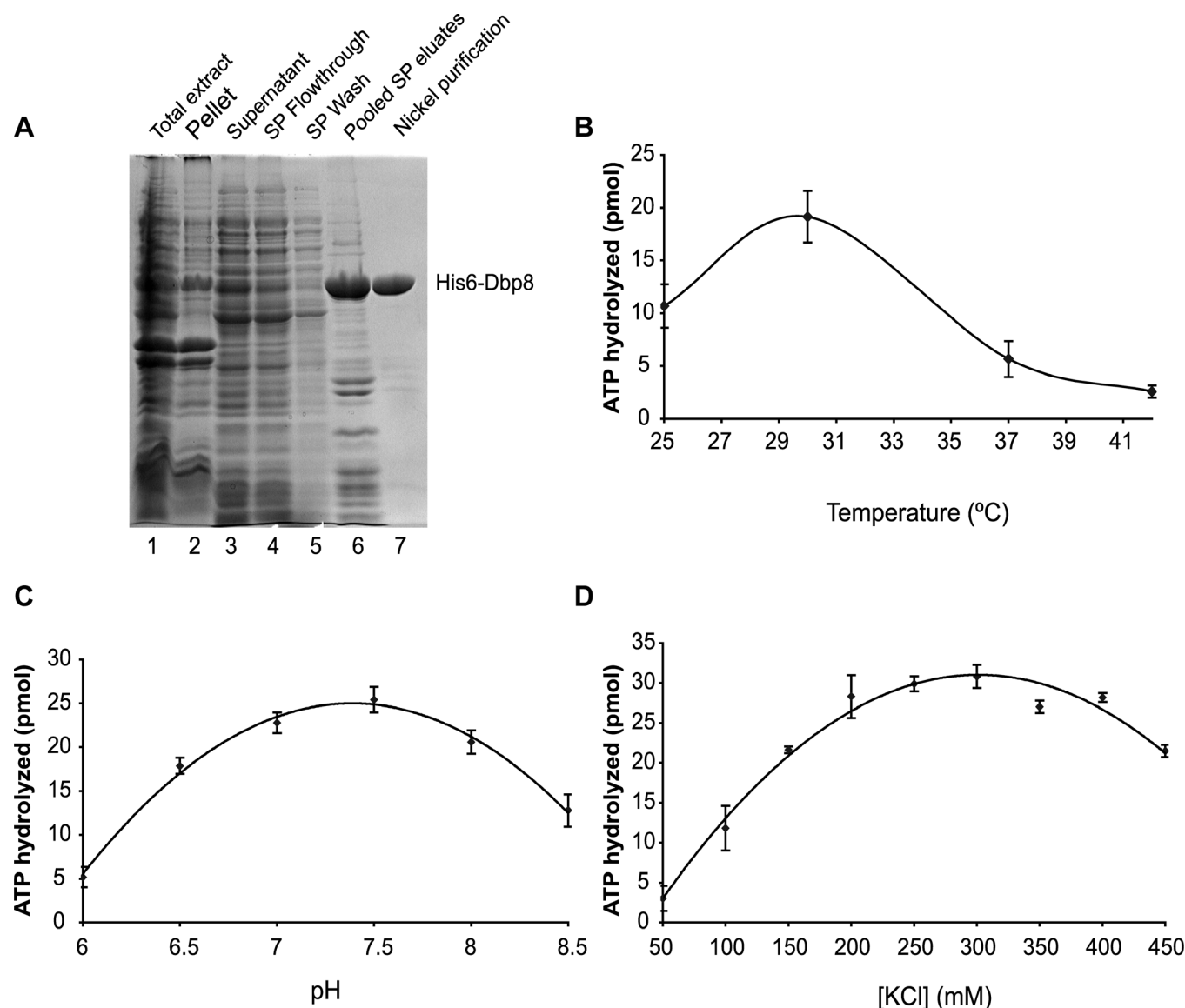


Figure 1. Purified recombinant Dbp8 has ATPase activity. (A) Purification of His6-Dbp8. Extracts prepared from *E. coli* expressing His6-Dbp8 (lane 3) was fractionated on a SP Sepharose cation exchange column. The column was extensively washed (lane 5) and proteins were eluted by applying a linear salt gradient. Fractions containing His6-Dbp8 were pooled (lane 6) and His6-Dbp8 was purified from these fractions to near homogeneity using Ni-NTA beads (lane 7). (B–D) Optimizing the temperature, pH and salt concentration for Dbp8 ATPase activity. ATP hydrolysis assays were performed at various temperatures (B); 25, 30, 37 and 42°C), different pH (C) and varying potassium chloride concentration (D) with 10 μ M ATP. ATP conversion (Y-axis) was calculated after 30 min by quantifying the phosphate release. Plotted are the averages and standard errors that were derived from three independent experiments.

either Coomassie blue staining or western blot analysis using an anti-six histidine antibody (Figure 2B). Little or no degradation products could be detected by either method and the mutations did not appear to dramatically affect the accumulation or stability of the protein (Figure 2B). Comparison of the ATPase activities of the wild-type and mutant proteins indicated that wild-type Dbp8 protein hydrolyzed significantly higher amounts of ATP than the Motif I (K52A and K52R) and Motif II mutants (D157A) (Figure 2C). Under the tested conditions, the D157A mutant had ~2% of the wild-type activity, whereas the Walker A mutations reduced ATPase activity roughly 10-fold. Thus, we conclude that the invariant Motif I lysine and the first aspartic acid in the Motif II are not only essential for cell

viability but also are required for Dbp8 ATP hydrolysis activity *in vitro*.

Dbp8 directly interacts with pre-rRNA processing protein Esf2

Other investigators had previously found that Dbp8 interacts with Esf2 in a large-scale yeast 2-hybrid screen (18). Esf2 is a nucleolar RRM-containing protein, which, like Dbp8, is required for processing at sites A₀, A₁ and A₂ in the pre-rRNA (19). The 2-hybrid interaction and the similar function in pre-rRNA processing suggested that they might function together. We have confirmed the predicted *in vivo* interaction through a directed yeast 2-hybrid assay (Figure 3A).

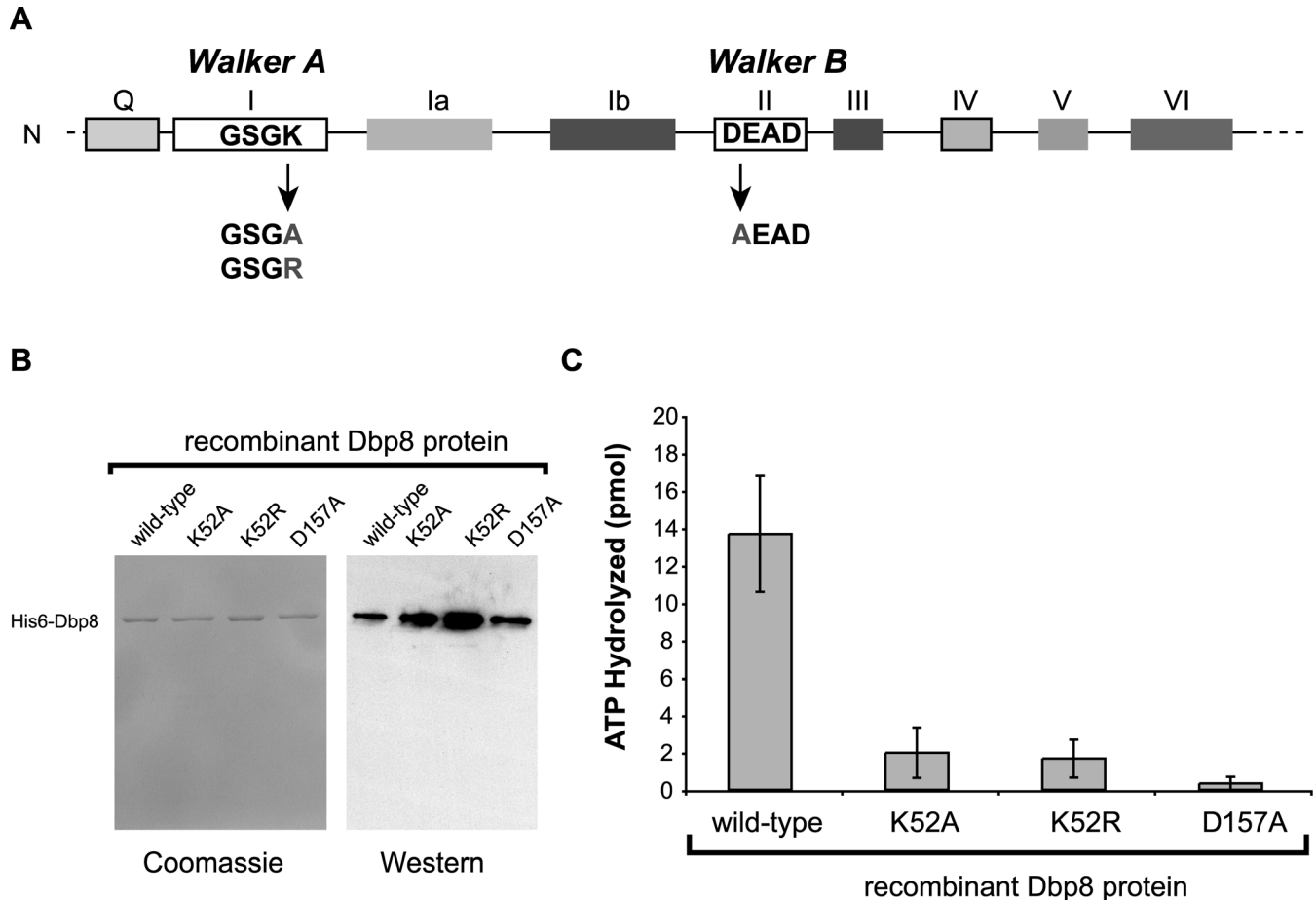


Figure 2. Dbp8 motif I and II mutants are defective in ATP hydrolysis *in vitro*. (A) Schematic representation of conserved motifs in Dbp8. The mutations that were introduced in motifs I and II are indicated by arrows. (B) SDS-PAGE analysis of purified His6-Dbp8 motif I and II mutants. Two μ g of each purified protein was resolved by SDS-PAGE. Proteins were either stained with Coomassie brilliant blue (left panel) or subjected to western blot analysis (right panel) using anti-His6 antibodies. (C) Motif I and II mutants are ATPase defective. ATP hydrolysis assays were performed with 10 μ M ATP, in the presence of 300 mM KCl and using 10 pmols of Dbp8 wild-type or motif I and II mutant proteins (X-axis). Mixtures were incubated for 30 min at 30°C and ATP conversion (Y-axis) was calculated after 30 min by quantifying the phosphate release. Plotted are the averages and standard errors that were derived from three independent experiments

To substantiate the *in vivo* interaction, we asked whether recombinant Dbp8 and Esf2 would directly interact *in vitro* (Figure 3B). Esf2, expressed and purified as a GST-fusion protein from bacterial extracts, was incubated with equimolar amounts of His6-Dbp8. Reconstituted complexes were precipitated using glutathione-Sepharose beads and bound proteins were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The results show that GST-Esf2 co-precipitated approximately stoichiometric amounts of Dbp8 (Figure 3B, lane 2). As negative controls we included GST alone, and two other recombinant proteins: His6-tagged Fap7 and GST-Rpa34 (Figure 3B, lanes 4–12). Fap7 is involved in 20S pre-rRNA processing but is not required for earlier processing steps and is therefore used as a negative control (24). Dbp8 was shown to co-purify with TAP-tagged Rpa34 (a subunit of RNA polymerase I) in affinity purification experiments (25) and GST-Rpa34 was therefore included to test a potentially direct interaction with His6-Dbp8. The results show that Esf2 (predicted pI \sim 8.4) did not co-precipitate His6-Fap7 (pI \sim 4.4; Figure 3B, lane 5). Furthermore, GST alone and GST-Rpa34 (pI \sim 9.5) did not

interact with Dbp8 (pI \sim 10) *in vitro* (Figure 3B, lanes 8 and 11), suggesting that the observed interactions between Esf2 and Dbp8 are not solely the result of non-specific electrostatic interactions. We conclude that Dbp8 directly and specifically binds Esf2 *in vitro*.

Finally, to confirm again if these proteins associate *in vivo* we performed co-immunoprecipitation experiments using strains in which Dbp8 was TAP-tagged and Esf2 was tagged with three copies of the HA tag (3HA). Extracts prepared from these strains were incubated with IgG Sepharose beads to immunoprecipitate Dbp8-TAP and co-precipitated proteins were analyzed by SDS-PAGE and western blot analysis using anti-HA antibodies (Figure 3C). We found that a small fraction of HA-tagged Esf2 associated with TAP-tagged Dbp8 in yeast extracts (Figure 3C, lane 6). As a positive control we used a strain in which two 66S pre-ribosome associated proteins were tagged (Rpf2-TAP, Rpf1-3HA; Figure 3C, lanes 1 and 2). As a negative control we used a strain in which only Esf2 was 3HA-tagged (Figure 3C, lanes 3 and 4). Thus, Esf2 directly binds Dbp8 *in vitro* and is complexed with Dbp8 *in vivo*.

Esf2 directly binds rRNA *in vitro*

Esf2 contains a predicted RRM RNA binding domain, and we therefore examined whether Esf2 would directly bind RNA (19). Using *in vitro* transcribed rRNA fragments (~200–300 nt in length), we sought to determine if GST-Esf2 binds rRNA *in vitro*. As TAP-tagged Esf2 associates with the 5'ETS of the pre-rRNA *in vivo* (19), we generated two radiolabeled rRNA transcripts that largely cover the 5'ETS region (Figure 3D). In addition, we made transcripts that contained 18S and 25S rRNA coding sequences (Figure 3D). Recombinant GST-Esf2 and GST alone (used as a negative control) were incubated with the various rRNA transcripts on ice to allow binding to occur and reconstituted complexes were precipitated using glutathione–Sepharose beads. Bound rRNAs were resolved by denaturing PAGE and detected by

autoradiography. The results demonstrate that GST-Esf2 is able to bind all the tested rRNA fragments although less efficiently to the 5'ETS 361–799 rRNA fragment (Figure 3D, lane 2). Furthermore, GST alone did not interact with any of the tested rRNA fragments (Figure 3D, lane 5). We conclude that recombinant GST-Esf2 binds RNA *in vitro*, albeit likely without any apparent sequence specificity.

Direct binding of Esf2 to Dbp8 stimulates Dbp8 ATPase activity *in vitro* likely by increasing its affinity for ATP

Since the two proteins interact, we asked whether Esf2 interaction with Dbp8 would stimulate its ATPase activity. We performed ATPase assays in which increasing amounts of Esf2 were added to the reactions, up to 1.5 M excess over His6-Dbp8 (Figure 4A). Because we initially could not detect a significant stimulation of ATPase activity by Esf2 in buffers containing 300 mM KCl, we performed the reactions with 200 mM KCl and indeed found a modest but significant increase in ATP hydrolysis with increasing Esf2. Under these conditions, addition of 1.5 M excess of GST-Esf2 to the reaction stimulated Dbp8 ATPase activity ~2-fold (Figure 4A). In contrast, the presence of 1.5 M excess of GST-Rpa34 did not have a stimulatory effect on Dbp8 ATPase activity. GST-Esf2 and GST-Rpa34 alone showed only residual background activity, usually consisting of hydrolyzing 1–5% of the total ATP. For comparison, background hydrolysis of ATP in buffer during the reaction times was usually about 1 or 2% (data not shown).

Surprisingly, when we analyzed Dbp8 ATPase activity in the presence of GST-Esf2 over varying KCl concentrations (50–450 mM; Figure 4B), the highest Dbp8 ATPase activity

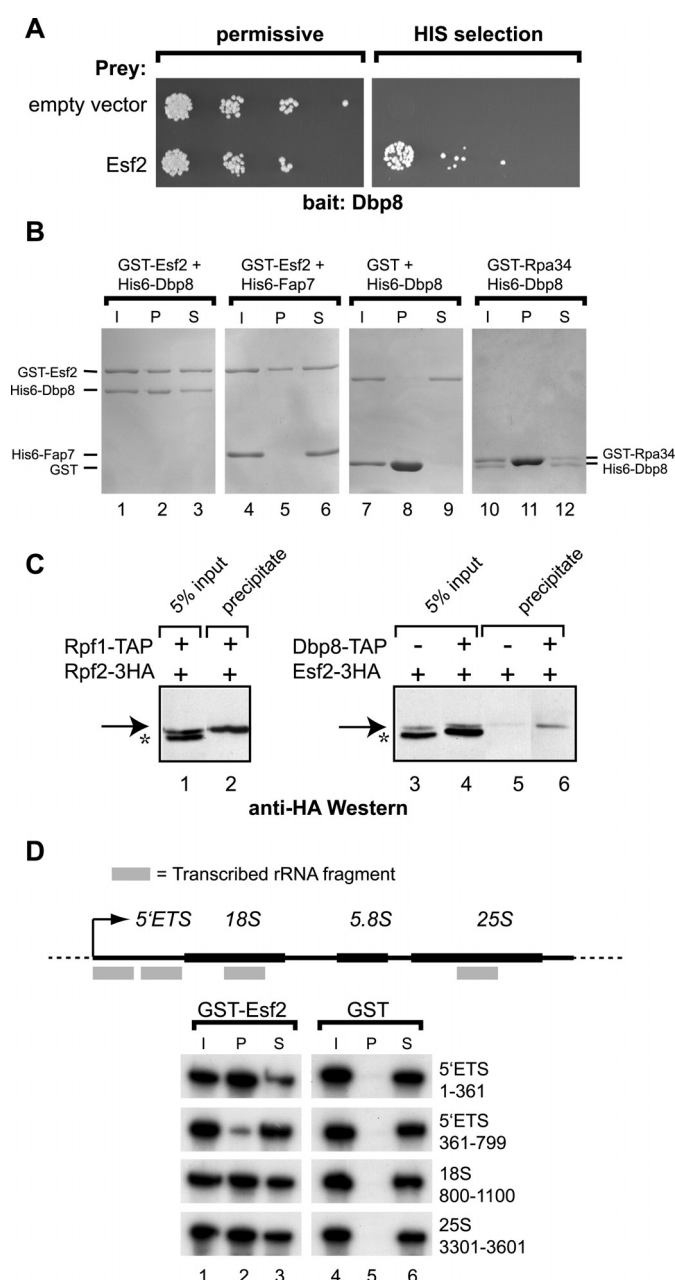


Figure 3. Dbp8 directly interacts with the RNA binding protein Esf2 *in vivo* and *in vitro*. (A) Dbp8 interacts with Esf2 in a yeast 2-hybrid assay. The yeast 2-hybrid host strain carrying the Esf2 bait vector and either Dbp8 prey vector or empty prey vector were serially diluted and tested for growth on permissive (+His) or selective media (–His selection). (B) Recombinant Dbp8 directly binds GST-Esf2 in GST pull-down assays. His6-Dbp8 was mixed with equimolar amounts of GST, GST-Esf2 or GST-Rpa34 and incubated on ice for 1 h. As an additional negative control, a GST pull-down assay was performed with GST-Esf2 and His6-Fap7 (24). GST-fusion proteins were precipitated using glutathione–Sepharose beads and bound proteins (‘P’; lanes 2, 5, 8 and 11) were resolved by SDS–PAGE and stained with Coomassie brilliant blue. Ten percent of the input material (‘I’; lanes 1, 4, 7 and 10) and 10% of the supernatants (‘S’; lanes 3, 6, 9 and 12) was also analyzed. (C) Esf2 associates with Dbp8 *in vivo*. Strains expressing various TAP and/or 3HA-tagged proteins (indicated on top by + or – signs) were grown in YP media to exponential phase. Extracts prepared from these strains were incubated with IgG beads for 1 h at 4°C. Immunoprecipitated proteins were separated by 10% SDS–PAGE and 3HA-tagged proteins were detected by western blot using mouse monoclonal anti-HA antibodies (12CA5; lanes 2, 5 and 6). As a positive control, immunoprecipitations were performed with IgG beads using a strain in which two pre-66S associated proteins were tagged (Rpf1-TAP and Rpf2-3HA; lane 2). As a negative control, a strain was used in which only Esf2 was 3HA-tagged (lane 5). Five percent of the amount of extract used for the immunoprecipitation was also analyzed (lanes 1, 3 and 4). The asterisk indicates a yeast protein that is non-specifically recognized by the anti-HA antibody. (D) GST-Esf2 directly binds RNA *in vitro*. GST-Esf2 or GST alone were incubated with various radiolabeled *in vitro* transcribed rRNA fragments that contained 5'ETS, 18S or 25S sequences (as illustrated). Complexes were precipitated using glutathione–Sepharose beads and bound RNAs were resolved by 8% denaturing PAGE and visualized by autoradiography (lanes 2 and 5). Ten percent of the input material (‘I’; lanes 1 and 4) and 10% of the supernatants (‘S’; lanes 3 and 6) were also analyzed.

was measured at 50 mM KCl and decreased with increasing KCl concentration. This was not due to lack of Dbp8-Esf2 association under the tested salt concentrations, as the *in vitro* binding of GST-Esf2 to His6-Dbp8 was not dramatically affected as judged by pull-down experiment with nickel-NTA beads (Figure 4B). These results suggest that Esf2 is a stimulatory factor for Dbp8 ATPase activity.

To determine the mechanism of how binding of Esf2 to Dbp8 could stimulate its ATPase activity, we determined Michaelis–Menten kinetic parameters in the presence or absence of Esf2. ATP hydrolysis was measured at varying ATP concentrations (10–500 μ M) and in the presence or absence of equimolar amounts of Esf2. Initial velocities (v) were calculated after 3 min of incubation at 30°C. During this time period we observed a linear correlation between

time and the amount of ATP hydrolyzed (data not shown). Average velocities were fitted to the Michaelis–Menten equation and K_M (ATP) and k_{cat} values were calculated (Figure 4C). When bound to Esf2, Dbp8 has a K_M for ATP of 86 μ M, \sim 2-fold lower than the K_M of Dbp8 alone (194 μ M). Under the tested conditions, Dbp8 binding to Esf2 increased the k_{cat} from 17 min^{-1} to 52 min^{-1} . Thus, Esf2 stimulates Dbp8 ATP hydrolysis activity likely by increasing its affinity for ATP, thereby increasing its ATPase activity.

The C-terminal domain of Esf2 is required for binding to Dbp8 and for stimulating Dbp8 ATPase activity

If Esf2 is indeed a co-factor for Dbp8 enzymatic activity, Esf2 binding to Dbp8 should be required for function. Bioinformatics searches for conserved protein motifs in Esf2 indicated that it had two coiled-coil domains (40–67 and 225–254 amino acids) on either side of the RRM (98–196 amino acids). Since coiled-coil domains can function as protein–protein interaction scaffolds [reviewed in (26)], we predicted that one of these domains might be involved in Dbp8 binding. To test this, we generated a number of Esf2 deletion mutants (Figure 5A) and assayed their ability to interact with Dbp8 in a directed yeast 2-hybrid assay (Figure 5B). Deletion of the first 90 amino acids, including the first coiled-coil domain, (Esf2 Δ N) or deletion of the predicted RRM motif (Figure 6A; 91–205 amino acids; Esf2 Δ RRM) did not noticeably impair interaction with Dbp8. In contrast, the C-terminal deletion mutant (Figure 5A; Esf2 Δ C) failed to interact with Dbp8 in the 2-hybrid assay (Figure 5B), suggesting that amino acids 206–317, which include one of the coiled-coil domains, are required for binding to Dbp8. When tested in the ATP hydrolysis assay, the Esf2 Δ C mutant, which did not bind Dbp8, also no longer substantially stimulated Dbp8 activity (9-fold for Esf2 WT versus 2-fold for Esf2 Δ C; Figure 5C). Therefore, Esf2

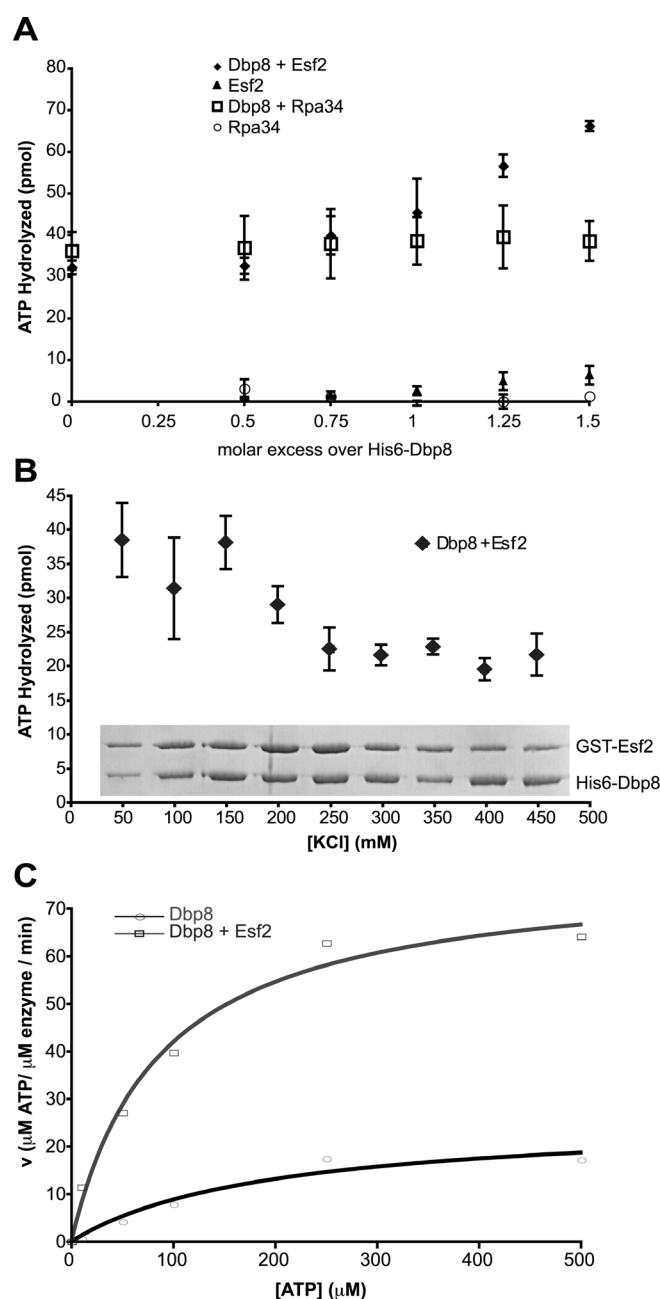


Figure 4. (A) Esf2 stimulates Dbp8 ATP hydrolysis activity *in vitro*. A total of 10 pmol of Dbp8 was incubated with various amounts of GST-Esf2 or GST-Rpa34 (0–1.5 M excess over Dbp8, X-axis) in buffers containing 200 mM KCl, for 25 min on ice to allow complex formation to occur. ATP was then added to the reaction and ATP hydrolysis (Y-axis) was measured after 30 min of incubation at 30°C. Plotted are the averages and standard errors that were derived from three independent experiments. (B) Esf2-mediated stimulation of Dbp8 ATPase activity is optimal at lower potassium chloride concentrations. ATP hydrolysis assays were performed using 5 pmol of recombinant Dbp8 and GST-Esf2 and varying salt concentrations (50–450 mM KCl). ATP hydrolysis was measured after 30 min of incubation at 30°C. Plotted are the averages and standard errors that were derived from three independent experiments. To determine if under these conditions GST-Esf2 and Dbp8 still form a complex, 300 pmols of each protein were incubated under the same conditions used for the ATP hydrolysis assay. Protein complexes were precipitated using Ni-NTA beads, resolved by SDS-PAGE and stained with Coomassie brilliant blue [Figure embedded in the graph shown in (B)]. (C) Dbp8 binding to Esf2 increases Dbp8 affinity for ATP and increases the ATP turnover rate. ATP hydrolysis assays were performed using 5 pmols of Dbp8 in the presence or absence of equimolar amounts of Esf2 in 50 mM KCl and various ATP concentrations. The initial velocities, plotted on the Y-axis, were determined by measuring the amount of ATP hydrolysis (μ M) during a 3 min period (data not shown) in the presence of various [ATP] (10, 50, 100, 250 and 500 μ M). The data were fitted to the Michaelis–Menten equation from which the K_M and k_{cat} were determined. Plotted are the averages derived from two independent experiments.

binding to Dbp8 is required for its stimulatory effect, and the C-terminal domain of Esf2 is required for Dbp8 binding.

Finally, we determined whether the mutant Esf2 proteins were functional *in vivo*. Since Esf2 is essential for viability,

we tested whether the deletion mutants could support growth using a strain where the endogenous protein was under the control of a galactose-inducible/dextrose repressible promoter (*GAL::3HA-ESF2*). Serial dilutions of cells expressing the wild-type or mutant Esf2 proteins or carrying empty vector (p415GPD) were spotted on galactose based solid medium (to verify even spotting of the culture) and plates containing dextrose media (to deplete endogenous Esf2). The results show that the Esf2 Δ C and Δ RRM mutants were unable to support growth in cells depleted of endogenous Esf2 (Figure 5D). Interestingly, the N-terminal deletion did not appear to dramatically affect Esf2 function, since this mutant alleviated the growth defect in Esf2-depleted cells as well as the wild-type protein expressed from the plasmid.

Collectively, these results suggest that the C-terminal domain of Esf2 is essential for function, required for binding to Dbp8 and for stimulation of ATPase activity. In contrast, the N-terminal region flanking the putative RRM motif is neither required for Esf2 function nor binding to Dbp8.

5'ETS rRNA fragments stimulate Dbp8 ATPase activity *in vitro*

The association of Esf2 with the 5'ETS RNA *in vitro* and *in vivo* [this work, (19)], prompted us to investigate whether pre-rRNAs stimulate Dbp8 ATPase activity. We tested total yeast RNA (type III; Sigma) and the four rRNA transcripts that bound to GST-Esf2 in the *in vitro* binding studies (Figure 3D). We found only a small increase in Dbp8 ATPase activity (~ 1.35 -fold; Figure 6A) at 50 mM KCl with equimolar amounts GST-Esf2 and Dbp8 (1 μ M) in the presence of the 5'ETS rRNAs (0.8 μ M; 1–360, 361–799; Figure 6A). In contrast, total yeast RNA (1 mg/ml), the 18S rRNA and the 25S rRNA transcripts did not have any significant stimulatory effect under the tested conditions. To corroborate these results we performed titration experiments to determine the concentration of 5'ETS 0-360 RNA required for optimal Dbp8 ATPase activity and to test whether stimulation was dependent on the Esf2 co-factor (Figure 6B). Surprisingly, the best degree of stimulation by rRNA (6-fold under these conditions) occurred in the absence of Esf2 when equimolar

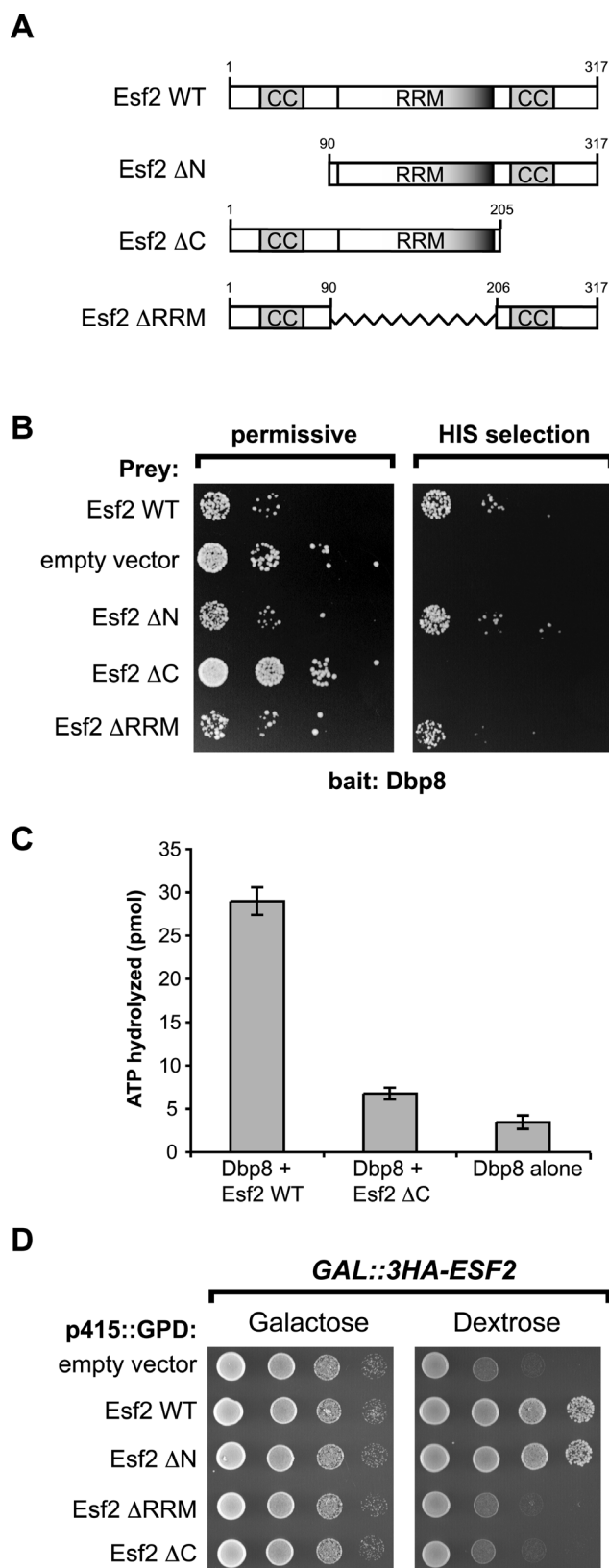


Figure 5. The essential C-terminal domain of Esf2 is required for binding to Dbp8 and stimulating its ATPase activity *in vitro*. (A) Schematic representation of the Esf2 wild-type (WT) and deletion mutants (Δ N, Δ C and Δ RRM) that were tested in the yeast two-hybrid screen for their association with Dbp8. The predicted RRM motif and coiled-coil domains are represented as boxes. The amino acid positions relevant to the deletions made are indicated. (B) The C-terminal domain of Esf2 is required for Dbp8 interaction in the yeast 2-hybrid assay. The yeast 2-hybrid host strain carrying either the Esf2 wild-type or Esf2 deletion mutants (bait) in combination with either Dbp8 (prey) or empty prey vector were serially diluted and tested for growth on permissive (+His) or selective media (His selection). (C) The C-terminal domain of Esf2 is required for stimulation of Dbp8 ATPase activity *in vitro*. ATP hydrolysis experiments were performed with 10 μ M ATP, 5 pmol of Dbp8 and 50 mM KCl, in the presence or absence (Dbp8 alone) of 5 pmol of GST-Esf2 wild-type (WT) or GST-Esf2 Δ C. ATP hydrolysis (plotted on the Y-axis) was measured after 30 min incubation at 30°C. Graphed are the averages and standard errors derived from three independent experiments. (D) The C-terminal domain of Esf2 is essential for function *in vivo*. Serial dilutions (10-fold) of *GAL::3HA-ESF2* strains carrying the empty vector or, p415GPD-*ESF2* wild-type and mutant alleles (Δ N, Δ RRM and Δ C) were grown in synthetic galactose media (SG/R-LEU) and spotted on either galactose containing plates (left panel; SG/R-LEU) or galactose containing plates (right panel; SD-LEU).

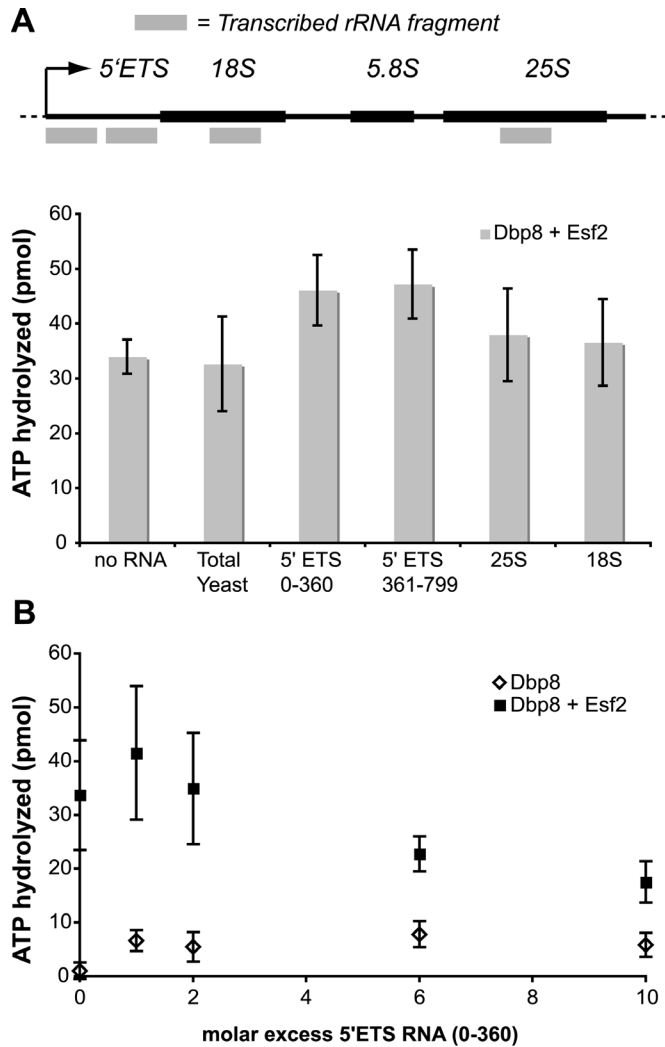


Figure 6. Pre-rRNA fragments stimulate Dbp8 ATPase activity *in vitro*. (A) *In vitro* transcribed fragments of the 5'ETS rRNA stimulate Dbp8 ATP hydrolysis in the presence of Esf2. ATP hydrolysis assays were carried out with 10 μ M ATP, 10 pmol of Dbp8 and 10 pmol of Esf2, 1 μ g/ μ l of total yeast RNA, 0.8 μ M of ribosomal RNAs (schematically outlined on top of the graph) and 50 mM KCl. Samples were incubated at 30°C for 30 min. Plotted are the averages and standard errors derived from three independent experiments. (B) *In vitro* transcribed 5'ETS rRNA fragments stimulate Dbp8 ATP hydrolysis in the absence of Esf2. ATP hydrolysis experiments were performed with 10 μ M ATP, 5 pmol of Dbp8 and 50 mM KCl, in the presence or absence of equimolar amounts of Esf2 and various amounts of 5'ETS rRNA (0–360 fragment; plotted on the X-axis). ATP hydrolysis (plotted on the Y-axis) was measured after 30 min incubation at 30°C. Plotted are the averages and standard errors derived from three independent experiments.

amounts of the 5'ETS 0-360 rRNA transcript was added to the reaction (Figure 6B). However, while rRNA was stimulatory for ATPase activity, the effect is small compared to the stimulatory effect of the Esf2 co-factor (30-fold under these conditions).

DISCUSSION

We have characterized the *in vitro* enzymatic activity of Dbp8, a DEAD box helicase required for small ribosomal subunit biogenesis (13). We identify the RRM-containing

protein, Esf2, also required for small ribosomal subunit biogenesis (19), as a Dbp8 binding protein that acts as an auxiliary factor to stimulate Dbp8 ATPase activity. The C-terminal domain of Esf2 is required for Dbp8 binding, for stimulation of ATP hydrolysis and for its *in vivo* function. We show that Esf2 also binds promiscuously to RNA, and that while some rRNA fragments stimulate Dbp8 ATPase activity, Esf2 is a more potent stimulator of ATPase activity than RNA. We have thus shown that Esf2 is a co-factor for Dbp8 ATP hydrolysis.

Our findings indicate that Dbp8 is typical of a DEXD/H box RNA helicase with respect to its ATP hydrolysis activity [using Table 1 in Cordin *et al.* (6) as a basis for comparison]. It differs from most other DEXD/H box RNA helicases characterized *in vitro* in that, under the conditions that we have used, its ATPase activity does not require added RNA. There is, however, a slight stimulation of ATPase activity when *in vitro* transcribed rRNA is added. Notably, of the five other DEAD box helicases required for ribosome biogenesis that have been studied *in vitro*, three do not require RNA for ATPase activity (Dbp4, Dbp9 and Rok1), one requires it (Has1), and Rrp3 was not studied in enough detail (14–17,27). In contrast, the bacterial DEXD/H box protein DbpA, also involved in ribosome biogenesis, has an absolute requirement for a specific portion of the 23S rRNA for *in vitro* ATP hydrolysis (28,29).

Since DEXD/H box RNA helicases generally participate in cellular processes that require RNA–protein complexes, it is not surprising that many DEXD/H box helicases have known interacting proteins (9) and in some cases a single helicase can have multiple interacting proteins. New partners for cellular helicases continue to be identified, including ones from human viral pathogens, such as cytomegalovirus (CMV) (30). In general, protein co-factors are thought to increase the ATPase and unwinding activities of their associated helicase. They can also have other effects. For example, the interaction of the NS4A protein of hepatitis C virus with the viral DEXD/H helicase NS3 changes the helicase substrate specificity from DNA to RNA (31). In addition, an RNA helicase can act as an RNA chaperone, as in the case where the helicase CYT19 recruits CYT18 (tyrosyl-tRNA synthetase) to splice the group I intron in yeast mitochondria (32).

However, of the studied interactions, there are only a handful of examples where the mechanism of the effect of the protein co-factor on helicase enzymatic properties has been elucidated *in vitro*. The eukaryotic translation factor DEXD/H box RNA helicase eIF4A has several co-factors, of which eIF4B and eIF4G are the most well-studied. Both stimulate ATPase activity and RNA unwinding (33–37). As with the effect of Esf2 on Dbp8, addition of the eIF4B co-factor to the helicase eIF4A increases its affinity for ATP (38). Similarly, RhlB, involved in RNA degradation as part of the *E.coli* degradosome, interacts with RNase E, which stimulates its ATPase activity (39).

What is the mechanistic role of Esf2 in enhancing the ATPase activity of Dbp8? Esf2 binding to Dbp8 may stabilize the tertiary structure of Dbp8 or induce a conformational change that is optimal for Dbp8 activity. A similar mechanism has been proposed for the translation initiation factors eIF4A and its co-factor eIF4G, two proteins that are part of the eIF4F complex involved in recruiting the small ribosomal

subunit to the 5' end of mRNA during translation (33,34). Based on solution structure studies, it has been proposed that binding of eIF4G to the C-terminal domain of eIF4A enhances the enzymatic activities by stabilizing an orientation of the two globular helicase domains that supports the 'active' conformation of eIF4A, a model that has been referred to as the 'soft clamp' model (34). Thus, it is tempting to speculate that Esf2 regulates Dbp8 activity by inducing a conformation that switches Dbp8 into its active state.

Although we have demonstrated ATPase activity for Dbp8, we have not been able to show ATP hydrolysis-dependent strand separation (also sometimes referred to as 'RNA unwinding'). Using RNA substrates with 12 bp duplexes, we could frequently detect strand separation of substrates containing 3' overhangs, but not blunt or substrates with 5' overhangs (S. Granneman, C. Y. Lin and S. J. Baserga, unpublished data). Unfortunately, this activity was also regularly observed in the absence of ATP and with an ATPase defective Dbp8 mutant protein, suggesting that binding of Dbp8 to the substrate with the 3' overhang was sufficient to separate the strands. Thus, we have been unable to unequivocally demonstrate energy dependent RNA unwinding or strand separation by Dbp8, and have therefore not been able to adequately test the role of Esf2 in this process.

Previous experiments have shown that Dbp8 and Esf2 are protein components of the large RNP, the SSU processome [also called 90S pre-ribosome; (19,40)], and as such are complexed with 40+ proteins and one or more snoRNAs. Consistent with SSU processome function, Esf2 has been found to be associated with 5'ETS rRNA fragments by immunoprecipitation (19). One intriguing possibility is that Esf2, as an RNA binding protein, acts to facilitate the interaction of Dbp8 with its RNA or RNP target. Thus, binding of Dbp8 to Esf2, possibly through its C-terminal coiled-coil domain, may not only activate Dbp8, but could also serve to place it near its site of action. Of note is that other studied DEXD/H box helicase co-factors that stimulate ATPase activity (eIF4B, eIF4G and RNase E) are also RNA binding proteins, indicating that this is likely to be a general theme among helicase co-factors.

Several possible functions have been ascribed to the RNA helicases involved in ribosome biogenesis. Because many have been found associated with large pre-ribosomal complexes, it has been suggested that they facilitate the numerous RNP remodeling steps, including disrupting rRNA-rRNA or protein-rRNA interactions, necessary for the maturation of ribosomal subunits. They could also facilitate rRNA-rRNA interaction, as helicases have been found to have an annealing activity (3). Another hypothesized function is that they unwind snoRNAs from the pre-rRNA after the chemical modification or cleavage steps have occurred. Consistent with this model, the DEXD/H box RNA helicase Dbp4 has recently been shown to be required for the release of the U14 snoRNA from the pre-rRNA, although it remains unclear whether it is Dbp4 that unwinds the duplex (15). In all cases, the natural RNA or RNP substrates of the helicases involved in ribosome biogenesis have unfortunately remained elusive (11,12,15,41,42). It is probable that, like Dbp8, the seventeen other DEXD/H box helicases required for ribosome biogenesis carry out their function in rRNA unwinding, RNP remodeling or rRNA annealing in conjunction with a specific

co-factor that stimulates their activity and perhaps directs them to their target substrate.

ACKNOWLEDGEMENTS

The authors are grateful to Wendy Bussen, Stephen van Komen, Margaret Macris, Michael Sehorn and Patrick Sung for help and advice on protein purifications and ATP hydrolysis assays and providing reagents and their facilities. The authors are grateful to Amanda Solem, Olga Federova and Anna Marie Pyle for their advice and for providing reagents and their facilities. This work was supported by Leslie H. Warner and Anna Fuller Postdoctoral Cancer Research fellowships (S.G.), Arnold and Mabel Beckman Foundation (C.Y.L.) and the National Institutes of Health to S.J.B. (GM52581). Funding to pay the Open Access publication charges for this article was provided by NIH GM52581.

Conflict of interest statement. None declared.

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